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In Vitro Frog Sciatic Nerve as a Peripheral Nerve Model for Studies of the Mechanism of Action of Low Energy Lasers: Part One

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Background and Objective: **There have been numerous reports of modulation of peripheral nerve action potential characteristics through application of low energy laser irradiation (LELI), although no mechanism has yet been advanced to explain these observations. In order to investigate the mechanism of LELI effects in peripheral nerve tissue, a well-characterized, reliable, and robust peripheral nerve preparation is required. The objective of this study was to evaluate the in vitro frog sciatic nerve as a candidate model for future LELI mechanism studies.**
Materials and Methods: **Following 60-minute baseline recordings of compound action potential (CAP) amplitude, latency, depolarization rate, and repolarization rate, helium-neon (HeNe) laser irradiation (632 nm, 15 min, 1–7 J, 44–320 J/cm^2) was delivered to one of two sites on the nerve. Laser-induced changes in CAP parameters were analyzed during irradiation and for 60 minutes post-irradiation using a repeated measures linear regression model.**
Results: **In the treatment group that received 7 J of HeNe energy over the recording electrode, CAP latency increased relative to nonirradiated controls during the postirradiation period. No other treatment group demonstrated laser-induced changes in CAP characteristics at any time during the experiment.**
Conclusion: **HeNe irradiation demonstrated limited ability to alter the CAP under these conditions. As such, the in vitro frog sciatic nerve is an inappropriate model for mechanism of action studies. Lasers Surg. Med. 21:32–41, 1997** **© 1997 Wiley-Liss, Inc.**

Key words: biostimulation; HeNe laser; low intensity laser therapy; repeated measures linear regression analysis

INTRODUCTION

A sizable body of literature exists concerning the use of low energy laser (or light) irradiation (LELI) to enhance wound healing, pain relief, and nerve repair and regeneration [for reviews, see 1–5]. There have been several reports of enhancement of function of healthy and injured peripheral nerves through transcutaneous [6–11] and direct [12–14] LELI, although no mechanism has yet been advanced to explain this phenomenon. One of the reported findings of Rochkind et al. [6–11] in the 1980s was that transcutaneous helium-neon (HeNe) laser irradiation of in vivo noninjured rat sciatic nerves strongly enhanced compound action potential (CAP) amplitude immediately after initiation of irradiation, an effect that persisted up to 360 days postirradiation. Direct HeNe laser irradiation of injured [14] and healthy [12] rat sciatic nerves also reportedly increased CAP amplitude.

Although successful modulation of peripheral nerve function with LELI has been frequently reported, investigation of the mechanism

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of action of LELI in this tissue has lagged far behind. One component necessary to investigate rigorously the mechanism of light action on peripheral nerve function is a reliable and robust peripheral nerve preparation. An in vitro preparation represents a strong candidate model due to its inherent practical advantages such as convenient access to the tissue to accomplish precise irradiation and measurement protocols and metabolic isolation of the tissue from other, potentially confounding in vivo processes. Prior to initiating mechanism of action studies, however, it is a necessary first step to demonstrate that in vitro and in vivo peripheral nerve preparations respond similarly to LELI.

Few reports of LELI application to in vitro peripheral nerve preparations are available. Walsh et al. [13] investigated the effect of LELI on in vitro frog sciatic nerve CAP latency by using a pulsed 820 nm diode laser to irradiate nerves maintained in a nerve chamber. Delivery of 2.38–3.57 J of energy resulted in an increase in negative peak latency by 7 minutes after cessation of irradiation. These authors did not indicate the location at which laser irradiation was applied to the nerves relative to the site of CAP measurement, nor was a mechanism proposed to account for the observed increase in latency. Arber et al. [15] investigated HeNe irradiation effects on the in vitro rat sciatic nerve using exposure times ranging from 10 seconds to 20 minutes (0.1–1 J/cm^2). No laser-induced alteration of CAP amplitude, latency, or rise time was evident.

The current work was prompted by the need to establish a reliable in vitro peripheral nerve model prior to undertaking an investigation of the mechanism of LELI action in this tissue. To accomplish this goal, the effect of HeNe laser irradiation was determined on in vitro frog sciatic nerves by irradiation under different energy delivery protocols at one of two sites relative to the site of CAP recording. Measured CAP parameters, including amplitude, latency, depolarization rate, and repolarization rate, were analyzed using a repeated measures linear regression analysis, a technique not previously applied in research of this sort, but one that offers advantages over discrete time point statistical methods frequently found in LELI literature. An account of the statistical methods used here and a full analysis of the ramifications of regression analysis as applied to LELI studies can be found in a companion work [16].

MATERIALS AND METHODS

Tissue Preparation

In accordance with an animal protocol approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee, large bullfrogs (5–6″ long) were immersed to their nostrils in a methanesulfonate solution ("MS-222", 1% w/v, Sigma Chemical Co., St. Louis, MO) for 10–15 minutes until a blink reflex, pinch reflex, or gular pumping were no longer noted. The sciatic nerve in each leg was exposed, excised, and laid without stretching over five silver wire electrodes in separate nerve chambers, as shown in Figure 1. Following tissue harvesting, the frog was pithed. Saline solution (0.9% w/v) was dripped onto the nerve at each point of contact with the electrodes to enhance electrical contact and was bubbled continuously in both covered chambers throughout the experiment to provide a humidified environment.

Irradiation Protocol

Following a baseline CAP recording period, 15 minutes of HeNe laser (632 nm, Spectra Physics Model 125) irradiation was delivered to the surface of one of the two nerves at the site in which the nerve contacted either the ground electrode or proximal recording electrode, depending on the treatment group in which the nerve was placed. The ground electrode was located ~5 mm proximal to the first recording electrode, as shown in Figure 1. The contralateral nerve in the other chamber was assigned to the nonirradiated control group.

A multimode optical fiber (0.6 mm core diameter) was used to deliver the laser energy to the selected location on the surface of the tissue. Laser power was attenuated to the desired level by incrementally altering the position of the optical fiber light acceptance cone relative to the beam. Laser power was measured with a calibrated power meter (Model 815, Newport Research) ~2 mm from the fiber output prior to and immediately after the irradiation period. The average of these two readings was recorded as the laser power for that nerve. The tip of the optical fiber was advanced through a small hole in the chamber lid and positioned within 2 mm of the tissue surface during irradiation.

CAP Recording Protocol

Throughout the experiment, each nerve was simultaneously stimulated orthodromically with

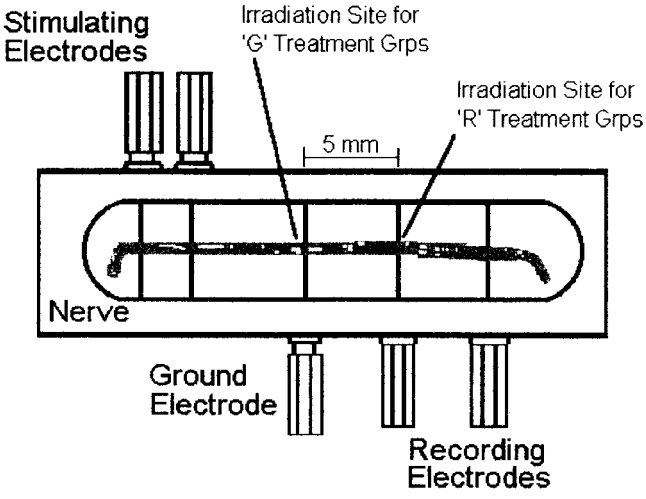

Fig. 1. Schematic of the nerve chamber used to evoke and record the frog sciatic nerve compound action potential (CAP). The CAP was evoked at the two proximal electrodes and recorded differentially over the two distal electrodes. The middle electrode was connected to ground. Laser irradiation was delivered at the surface of the nerve over either the ground electrode or the proximal recording electrode. Two nerve chambers were utilized simultaneously with one containing a control nerve while the other contained the contralateral irradiated nerve.

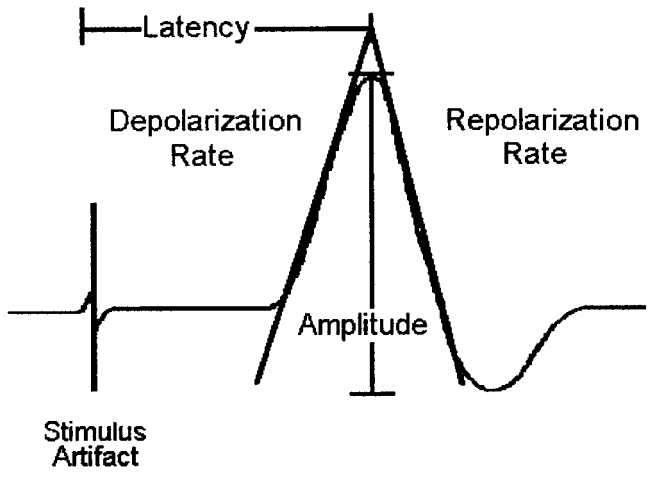

Fig. 2. A typical biphasic CAP. Four CAP parameters were measured as indicated: amplitude, latency, rate of CAP depolarization, and rate of CAP repolarization. Raw measurements were normalized by dividing by the pre-irradiation mean value of each CAP parameter.

two electrodes using a supramaximal stimulus (0.025 ms, 1.5 V). The resulting CAP was recorded differentially from the two most distal electrodes via a MacLab stimulator-A/D converter unit (Analog-Digital Instruments, Ltd., Australia) and saved on a computer. The middle electrode of each chamber was connected to a common ground.

For most preparations, only one biphasic action potential was evident corresponding to the combined contribution of the larger diameter and faster conducting fibers in the nerve. Infrequently, slower conducting fibers were also apparent in the recorded CAP as a separate peak with a longer latency than the primary peak. In these instances, CAP measurements were made using only the first negative peak. CAP amplitude was measured from peak to peak and latency measured from stimulus onset to the negative peak of the CAP, as shown in Figure 2. The rates of depolarization and repolarization of the evoked CAP were estimated by measuring the slope of the CAP trace on either side of the negative peak (Fig. 2). All measurements were made off-line using MacLab-supplied software.

The entire experiment, which lasted for 135 minutes, was performed in three consecutive phases. In phase 1, a CAP was simultaneously evoked and recorded in each nerve for 60 minutes at 1-minute intervals to establish the baseline CAP characteristics of each nerve. Following the baseline recording period, phase 2 was commenced, which consisted of 15 minutes of irradiation delivered to the nerves assigned to irradiated treatment groups. During phase 2, CAPs were simultaneously evoked and recorded in both irradiated and nonirradiated nerves at 1-minute intervals. Phase 3 consisted of a 60-minute post-irradiation period during which CAPs were stimulated and recorded at 1-minute intervals in both nerves. All experiments were conducted in a naturally lit room. No attempt was made to control or measure temperature changes in the nerve chambers or the tissue during the experiment.

Treatment Groups

Treatment groups in this study were designated according to the combination of mean total energy delivered to the tissue and site of irradiation as shown in Table 1. The desired total energy delivered to the tissue was determined through review of pertinent LELI literature. Although many previous LELI-peripheral nerve interaction studies suffer from incomplete documentation of experimental procedures and laser parameters, making replication of these studies impossible, analysis of available information indicated that most successful LELI applications delivered total energy of 0.13–15 J [6–11,12,14]. Of these studies, most found that energy levels <7 J were effective in altering CAP amplitude.

On the basis of this information, three en-

TABLE 1. Matrix of Treatment Groups

	Control	Group 1G	Group 4G	Group 4R	Group 7R
Energy delivered (J)*	0.0	0.96 ± 0.18	4.00 ± 0.10	3.87 ± 0.18	7.02 ± 0.41
Laser power (mW)*	0.0	1.07 ± 0.19	4.46 ± 0.08	4.31 ± 0.20	7.79 ± 0.43
Fluence (J/cm^2)*	0.0	44 ± 8	182 ± 5	176 ± 8	320 ± 19
# Nerves in treatment group	14	5	4	4	3
Site of irradiation	NA	Ground Electrode	Ground Electrode	Recording Electrode	Recording Electrode

*Mean ± SD.

ergy levels were chosen for this study: 1 J, 4 J, and 7 J. Since each irradiated nerve received 15 minutes of irradiation, laser power was attenuated appropriately to deliver the desired total energy to the tissue. Laser fluence or energy density was then approximated based on total energy delivered, the optical fiber core diameter, the optical fiber tip location 2 mm above the tissue, and a light divergence angle of 15° from the optical fiber tip.

Strict exclusion criteria were used to ensure that each sciatic nerve preparation exhibited stable CAP characteristics during the 60-minute baseline period. Nerve tissue preparations that demonstrated >±25% variation in CAP amplitude from the mean baseline amplitude for more than two consecutive minutes were excluded from the study. Nerves in which the evoked CAP became unrecordable at some time during the 135-minute recording period were also excluded from the study. The sample size listed in Table 1 for each treatment group refers only to nerves that met all criteria for inclusion in the study.

Statistical Analysis

By examining individual response profiles in each treatment group, it was determined that the optimum way to analyze the data was by separate linear regression models for each of the three experimental phases: preirradiation (Phase 1), irradiation (Phase 2), and postirradiation (Phase 3). A full account of the statistical procedures used in this study is given in a separate work [16]. Following is a brief overview of the data analysis techniques employed.

Normalized measurements were first computed by determining the mean value of each CAP parameter over the 60-minute pre-irradiation baseline period for each nerve. All subsequent raw CAP parameter measurements recorded in that nerve were then divided by the mean pre-irradiation value of the appropriate parameter in order to derive the normalized value of the CAP parameter corresponding to that time point in the given nerve. A random-coefficient linear regression analysis for unbalanced repeated measures was performed on the mean normalized CAP parameters for each phase-treatment group combination.

Due to the strong correlation of measurements made on the same nerve (e.g., correlation coefficient >0.91 for normalized amplitude), a first-order autoregressive covariance structure was fit to measurements made in the same phase of a treatment group. This covariance structure models the inter-measurement correlation as a function of the time lapse between individual measurements [16,17]. Further accounting for measurement correlation was accomplished by the use of a random-coefficient regression model.

Hypothesis testing was conducted on contrasts that measured differences in regression line slope between two phases in a treatment group relative to the difference in slopes in the control group between the same two phases: e.g., is the change in regression line slope between phase 1 and phase 3 in Group 1G different than the change in slope between phase 1 and phase 3 in the nonirradiated control group? The overall threshold for significance ($\alpha = 0.05$) was adjusted by the Bonferroni method to reflect multiple comparisons [18].

RESULTS

The effect of HeNe laser irradiation on CAP amplitude is demonstrated in Figure 3a–d. Laser irradiation under the experimental conditions described failed to induce any statistically significant change in CAP amplitude at any time in the experiment.

As demonstrated in Figure 4 a–d, HeNe laser irradiation was ineffective in altering CAP latency in three of the four treatment groups. In treatment group 7R shown in Figure 4d (i.e., 7.0 J of energy delivered over the proximal recording

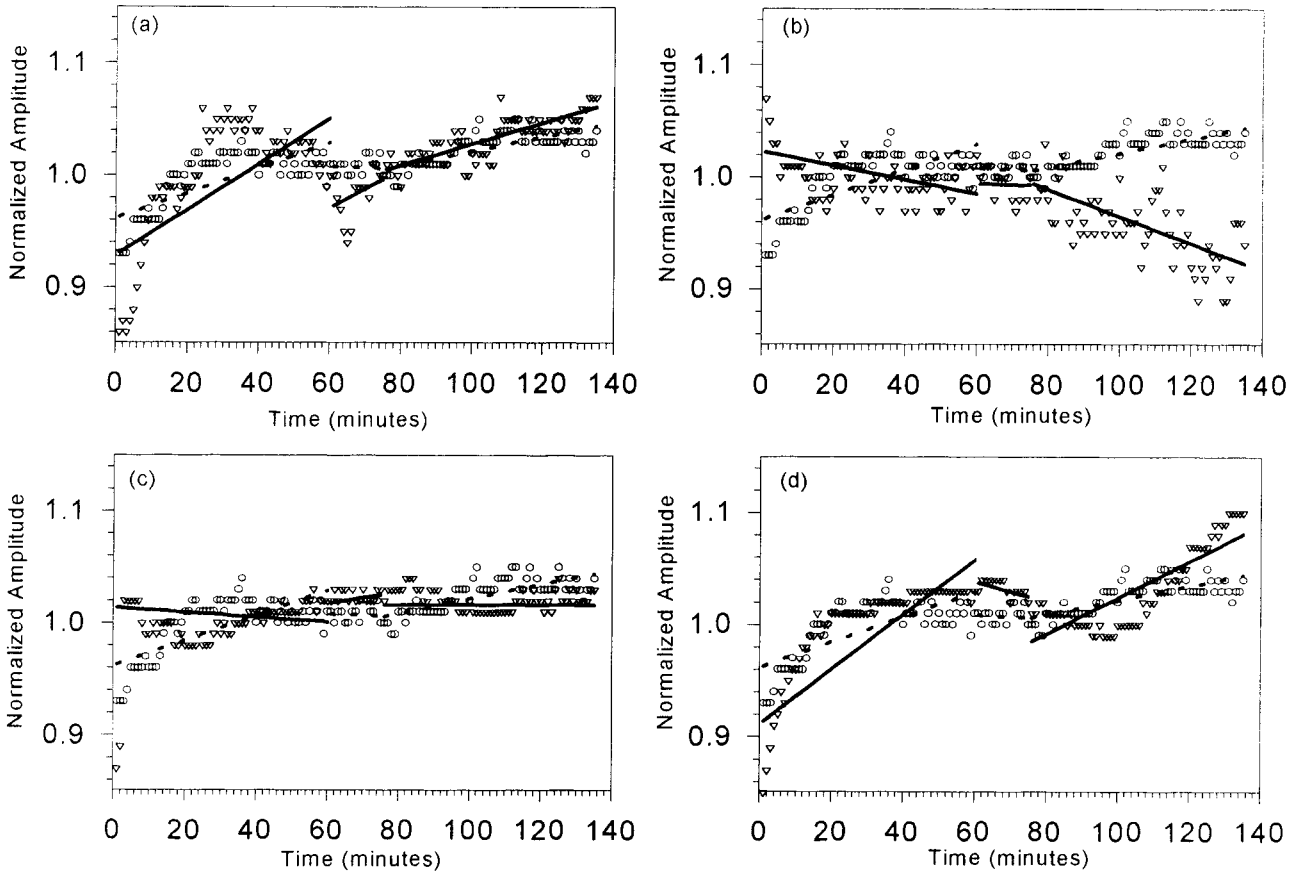

Fig. 3. Normalized amplitude in treatment groups 1 G **(a),** 4 G **(b),** 4 R **(c),** and 7 R **(d).** Mean values of nonirradiated (○) and irradiated (▽) CAP amplitude are plotted at 1-minute intervals. Regression lines computed for each phase are overlayed for the control group (------) and the irradiated group (——). HeNe laser irradiation was ineffective in altering CAP amplitude relative to nonirradiated nerves.

electrode), CAP latency demonstrated a statistically significant increase during the postirradiation phase compared to the nonirradiated control group over the same time period.

Figures 5a–d and 6a–d demonstrate the effect of the HeNe laser irradiation on the rate of depolarization and rate of repolarization of the frog sciatic nerve CAP, respectively. For these two CAP parameters, measurements were made every 5 minutes during Phases 1 and 3 and every 2 minutes during Phase 2 of the experiment. HeNe laser irradiation resulted in no statistically significant change in either rate of depolarization or rate of repolarization in any treatment group.

DISCUSSION

The results of the regression analysis of normalized CAP parameter measurements indicated that HeNe laser irradiation delivered to in vitro frog sciatic nerves had very limited effects on the evoked CAP. In particular, only latency was significantly altered under one irradiation protocol, 7 J delivered at the recording electrode, which appeared to cause an increase in latency relative to the control group. The increase in CAP latency or, equivalently, the decrease in nerve conduction velocity demonstrated in treatment group 7 R partially agrees with results reported by Walsh et al. [13], although the total energy delivered to the nerves differed in each case (2.38 J in Walsh's study and 7.02 J in the current study), the postirradiation recording period was much longer in this work (60 minutes compared to 7 minutes in Walsh's study), and the laser wavelength used was different (820 nm in Walsh's study compared to 632.8 nm in the current work). Walsh did not speculate on the mechanism responsible for the observed latency increase.

It is possible that the observed increase in CAP latency in Walsh's study [13] and the current work was actually due to compromised nerve vi-

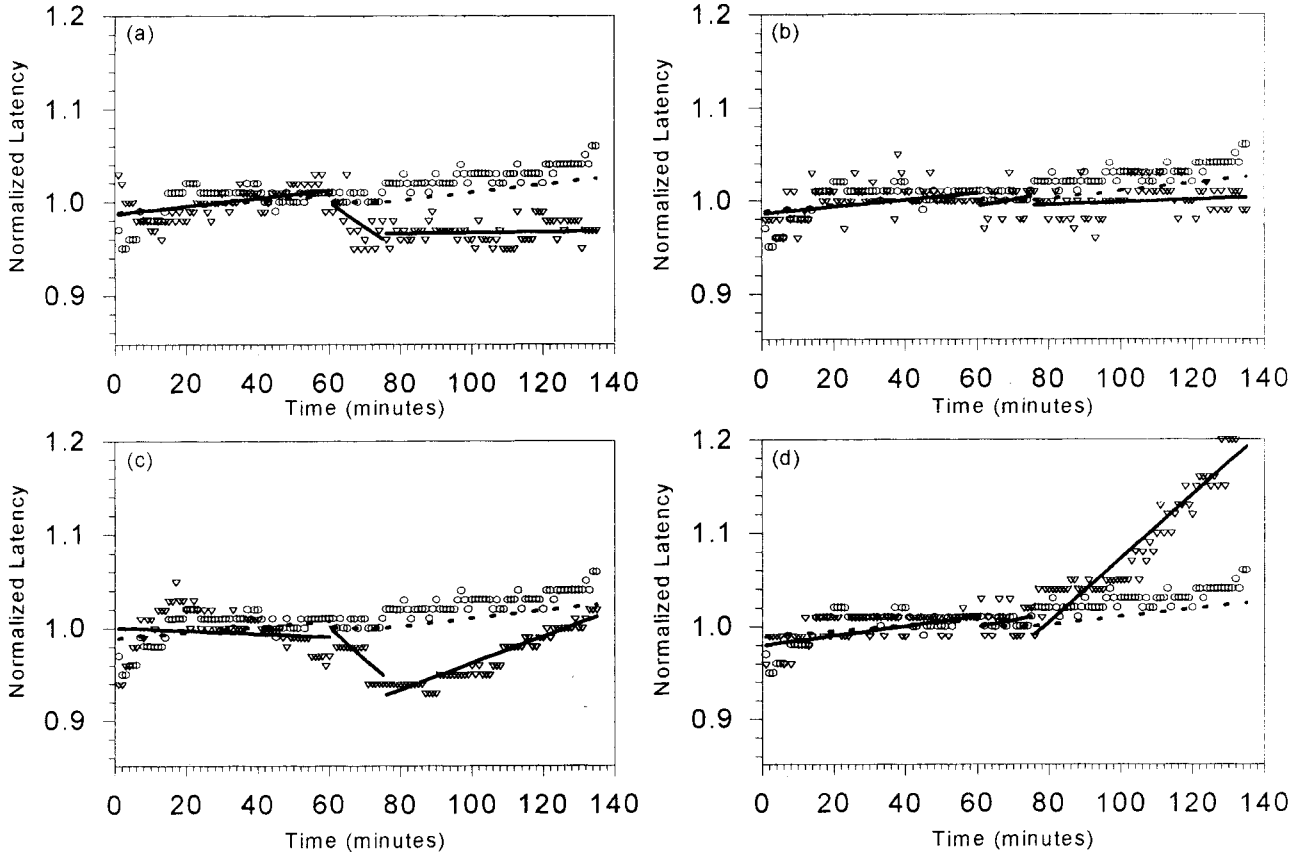

Fig. 4. Normalized latency in treatment groups 1 G **(a),** 4 G **(b),** 4 R **(c),** and 7 R **(d).** Mean values of nonirradiated (○) and irradiated (▽) CAP latency are shown with regression lines computed for each phase overlayed for the control group (------) and the irradiated group (——). HeNe laser irradiation significantly increased CAP latency in treatment group 7-R during the post-irradiation phase. No latency changes were noted in the other three treatment groups.

ability rather than the effect of laser irradiation. It was noted during the study that nerves that failed prior to the end of the experiment, and therefore did not meet the inclusion criteria, frequently demonstrated large, rapidly progressing increases in latency as their viability decreased, although CAP amplitude remained relatively constant. Since treatment group 7R contained only three nerves, each nerve exerted a large influence on the mean latency at each time point. Failing viability in one or more of the nerves likely contributed to increasing mean latency that had the effect of increasing the slope of the regression line fit to this data and resulted in the finding of statistical significance in this phase.

Although CAP latency was observed to increase in Group 7 R in the postirradiation period, possibly due to compromised tissue viability, CAP amplitude in the same treatment group during the same period did not change relative to the control group. This can be explained by considering the nature of the changes occurring in the nerve during generation and propagation of the action potential. There are at least two distinct scenarios by which an increase in latency may occur due to compromised viability without a corresponding decrease in CAP amplitude.

In the first scenario, the sodium and potassium gradients may have run down causing slight depolarization of the larger, myelinated axons, which are primarily responsible for generation of the observed CAP. Depolarization of an axon is known to partially inactivate sodium channels, which would have the effect of increasing threshold at the nodes of Ranvier. Because each nerve in this experiment was stimulated with a supramaximal stimulus, the increase in threshold at each node may not necessarily preclude CAP generation but would cause a slight reduction in conduction velocity since each node requires more time to reach threshold before regenerating the new action potential. The cumulative effect of the

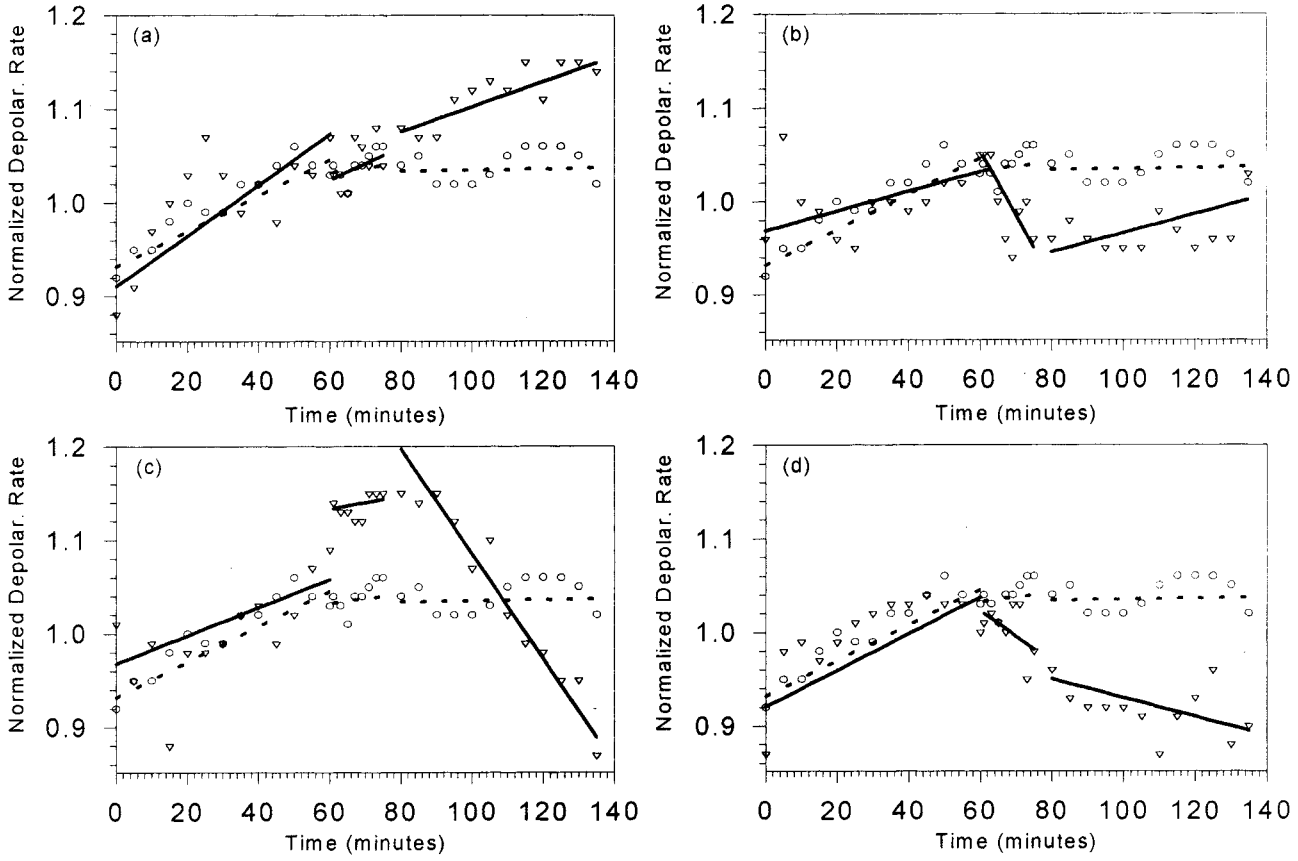

Fig. 5. Normalized rate of CAP depolarization in treatment groups 1G **(a),** 4G **(b),** 4R **(c),** and 7R **(d)**. Mean values of nonirradiated (○) and irradiated (▽) nerves are shown as are regression lines computed in each phase for the control group (----) and the irradiated group (——). HeNe laser was ineffective in altering this CAP parameter relative to nonirradiated nerves.

small delays in reaching threshold at each node would be observed distally as an increase in nerve latency without a change in CAP amplitude.

The second possibility that might account for the increased CAP latency without changes in CAP amplitude is that the in vitro conditions resulted in gradual changes in axonal membrane lipids, which altered the effective membrane capacitance or resistance. Small changes in either or both of these membrane properties would not be expected to significantly alter the amplitude of the CAP (although a change in CAP morphology may be observed). Conduction velocity may be slowed, however, by altering membrane resistance and capacitance.

The use of ineffective light dosages has been frequently suggested as a reason for the ineffectiveness of certain laser energy delivery protocols to alter CAP characteristics. An important question to be addressed in all LELI applications is how to determine effective energy doses for various tissues. In this study, the lack of success in altering CAP characteristics with HeNe laser irradiation may have been due in part to use of energy delivery parameters that were outside of the effective stimulatory range for this tissue. Unfortunately, this effective range of energy delivery parameters is not known for most tissues. A thorough review of pertinent LELI literature suggested that total energy levels <15 J delivered directly to the nerve tissue were generally effective in enhancing in vivo CAP amplitude. It is difficult, however, to define a suitable range of laser delivery parameters to ensure success, given the frequently incomplete reporting of experimental procedure that accompanies much of this literature. Often, attempts to derive the appropriate delivery parameters based on information in the literature yields improbable results.

For instance, in one report [12], it was asserted that CAP amplitude was enhanced in exposed rat sciatic nerves following irradiation with

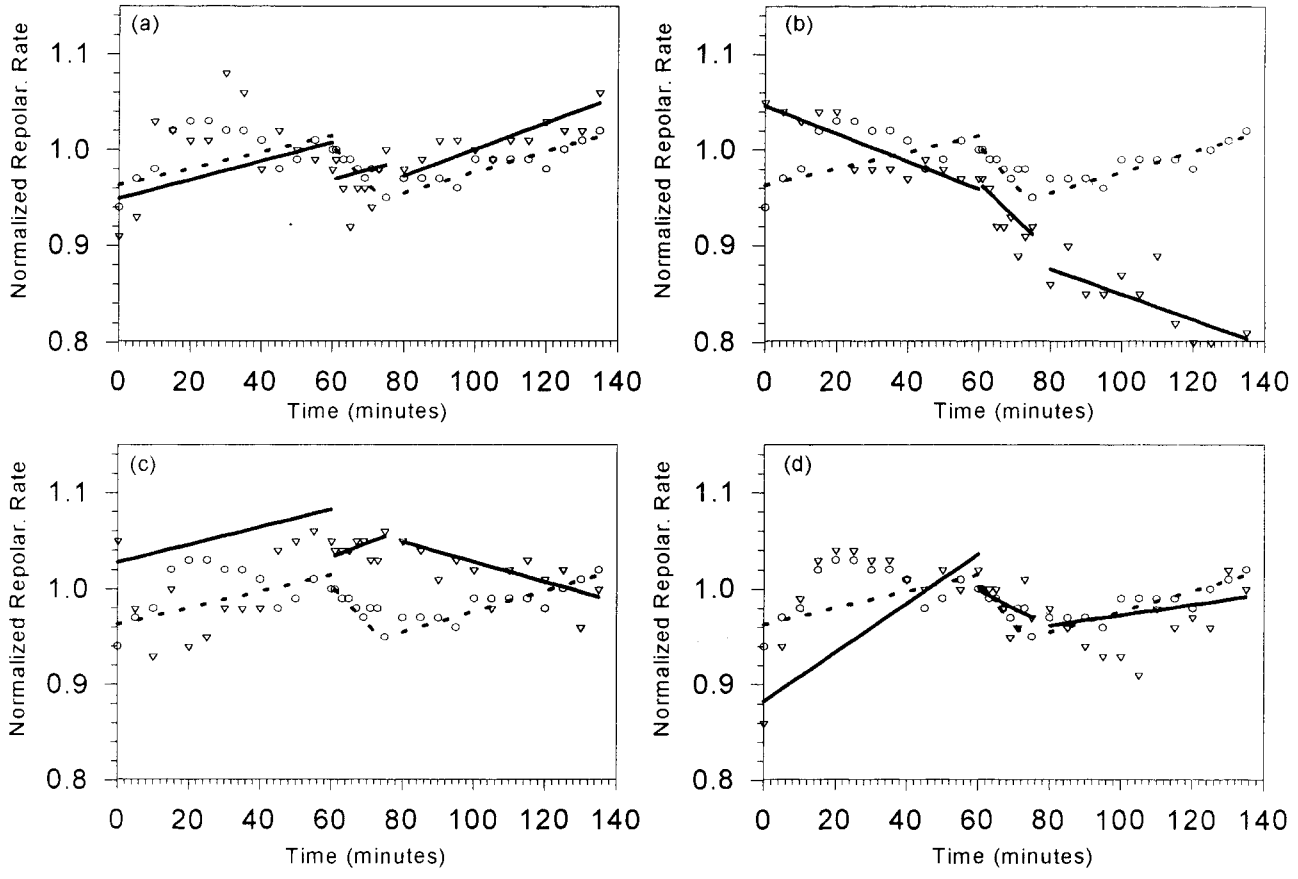

Fig. 6. Normalized rate of CAP repolarization in treatment groups 1 G **(a),** 4 G **(b),** 4 R **(c),** and 7 R **(d).** Mean values of nonirradiated (○) and irradiated (▽) nerves are shown as are regression lines computed in each phase for the control group (------) and the irradiated group (——). HeNe laser was ineffective in altering this CAP parameter relative to nonirradiated nerves.

a 0.3 mW HeNe laser for 7 minutes resulting in a fluence of 0.2 J/cm^2. Although the laser spot size was not reported, simple calculations indicate that the beam must have been defocused to 8.9 mm in diameter to achieve this fluence. In this most unlikely circumstance, only a small portion of the laser energy delivered to the tissue could have actually been absorbed by the sciatic nerve. In the same report, Rochkind et al. [12] suggested that HeNe laser fluences in the range 0.07–0.7 J/cm^2 were effective in altering CAP characteristics in the in vivo rat sciatic nerve. In the present study, calculated laser fluences were orders of magnitude greater than Rochkind's recommended range of 0.07–0.7 J/cm^2, even at the lowest power setting. It is not clear if the high fluences encountered in this current work resulted in laser delivery parameters outside of the effective range for this tissue, or if previous reports of fluence are erroneous, as demonstrated in the above example.

Another possible explanation for the inability of HeNe laser irradiation to alter CAP characteristics in this work involves differences in metabolic activity between in vitro and in vivo peripheral nerve preparations. In both cases, the ability to generate multiple CAPs remains intact. Generation and propagation of the CAP require the presence of sufficient adenosine triphosphate (ATP) stores in each axon to drive the transmembrane sodium-potassium pumps, which reestablish the ionic gradients in preparation for the next impulse. Application of low energy laser irradiation has been shown to alter ATP-production in vitro [19,20] and is a significant component of the currently accepted general mechanism of LELI action [21]. It is not clear, however, how laser-induced increases in ATP production may directly account for the enhancement of CAP amplitude reported in previous in vivo studies.

One difference between in vivo and in vitro nerve preparations is their ability to replenish these cellular ATP stores at the same rate. It may

not be possible to sufficiently enhance the formation of ATP in vitro, and therefore enhance the observed CAP, due to the reduced overall metabolic activity of the in vitro tissue, regardless of the effectiveness of the laser delivery parameters utilized. Other factors also represent obstacles to in vitro nerve function, including drying-induced structural changes and increased sensitivity to environmental temperature fluctuations. For these reasons, the in vitro frog sciatic nerve preparation, when used under the experimental conditions described in this study, represents an inappropriate tissue model for use in investigations of the mechanism of LELI action in peripheral nerve tissue.

CONCLUSIONS

HeNe laser irradiation delivered at two different sites was found to be ineffective in altering frog sciatic nerve CAP parameters in vitro. It is possible that the inability of the laser irradiation to effect the peripheral nerve tissue was primarily due to the in vitro experimental conditions. Although the frog sciatic nerve preparation was viable in vitro, the lack of active tissue metabolism might have been responsible for the failure of the laser irradiation to significantly alter physiological function. As such, an in vitro peripheral nerve model appears to be unsuitable for mechanism of LELI action studies.

In recent years, there has been a relative scarcity of new investigations into LELI-peripheral nerve tissue interaction, although there are numerous important questions that remain regarding the nature of the interaction of monochromatic light with this tissue. The first priority should be to determine the conditions necessary to consistently enhance CAP characteristics and to understand which delivery parameters are most important in influencing the nature of the CAP. It also would be useful to understand the effect of irradiation on other CAP characteristics such as threshold, refractory period, ionic conductance across axonal membranes and axonal recruitment, which have been ignored in past work. These studies should be conducted in vivo as it is unlikely that LELI is effective on in vitro peripheral nerve preparations. All of these factors, as well as complete reporting of pertinent energy delivery parameters, should be considered in future LELI studies.

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